

FORM PTO-1390 (REV 11-2000)	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 1721-29
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) <b>09/856707</b> Unknown
INTERNATIONAL APPLICATION NO. PCT/FR99/02949	INTERNATIONAL FILING DATE 29 November 1999	PRIORITY DATE CLAIMED 27 November 1998
TITLE OF INVENTION GP 120 MUTANTS AND BIOLOGICAL APPLICATIONS		
APPLICANT(S) FOR DO/EO/US VEAS et al.		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.</p> <p>4. <input checked="" type="checkbox"/> The U.S. has been elected by the expiration of 19 months from the priority date (Article 31).</p> <p>5. A copy of the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>a. <input type="checkbox"/> is attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> has been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US).</p> <p>6. <input checked="" type="checkbox"/> An English language translation of the PCT Request and the International Application as filed (35 U.S.C. 371(c)(2)).</p> <p>a. <input checked="" type="checkbox"/> is attached hereto.</p> <p>b. <input type="checkbox"/> has been previously submitted under 35 U.S.C. 154(d)(4).</p> <p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under <u>PCT Article 34</u></p> <p>a. <input type="checkbox"/> are attached hereto (required only if not communicated by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> have been communicated by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has <b>NOT</b> expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p> <p>8. <input checked="" type="checkbox"/> An English language translation of the amendments to the claims under PCT Article 34 is attached hereto.</p> <p>9. <input type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p> <p>10. <input checked="" type="checkbox"/> A English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p> <p><b>Items 11 To 20 below concern document(s) or information included:</b></p> <p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 C.F.R. 1.97 and 1.98.</p> <p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 C.F.R. 3.28 and 3.31 is included.</p> <p>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment.</p> <p>14. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</p> <p>15. <input type="checkbox"/> A substitute specification.</p> <p>16. <input type="checkbox"/> A change of power of attorney and/or address letter.</p> <p>17. <input type="checkbox"/> A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821-1.825.</p> <p>18. <input type="checkbox"/> A second copy of the published international application under 35 U.S.C. 154(d)(4).</p> <p>19. <input type="checkbox"/> A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).</p> <p>20. <input checked="" type="checkbox"/> Other items or information. Front page of the PCT Publication/ PTO-1449/ International Search Report</p>		

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) <b>09/836707</b> <small>Unknown</small>		INTERNATIONAL APPLICATION NO. <b>PCT/FR99/02949</b>		ATTORNEY'S DOCKET NUMBER <b>1721-29</b>	
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21. <input checked="" type="checkbox"/> The following fees are submitted:				<b>CALCULATIONS</b> PTO USE ONLY							
<b>BASIC NATIONAL FEE (37 C.F.R. 1.492(a)(1)-(5)):</b> -- Neither international preliminary examination fee (37 C.F.R. 1.482) nor international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO .....\$1000.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$860.00 -- International preliminary examination fee (37 C.F.R. 1.482) not paid to USPTO but international search fee (37 C.F.R. 1.445(a)(2)) paid to USPTO .....\$710.00 -- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00 -- International preliminary examination fee (37 C.F.R. 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00  <div style="text-align: right;"><b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b></div>				<table border="1" style="width:100%; border-collapse: collapse;"> <tr> <td style="width:10%;">\$</td> <td style="width:40%;">860.00</td> <td style="width:50%;"></td> </tr> <tr> <td>\$</td> <td>130.00</td> <td></td> </tr> </table>		\$	860.00		\$	130.00	
\$	860.00										
\$	130.00										
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input checked="" type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(e)).											
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE								
Total Claims	14	-20 =	0	X	\$18.00						
Independent Claims	2	-3 =	0	X	\$80.00						
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)					\$270.00						
<b>TOTAL OF ABOVE CALCULATIONS =</b>					<b>\$ 990.00</b>						
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.					0.00						
<b>SUBTOTAL =</b>					<b>\$ 990.00</b>						
Processing fee of \$130.00, for furnishing the English Translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 C.F.R. 1.492(f)).					0.00						
<b>TOTAL NATIONAL FEE =</b>					<b>\$ 990.00</b>						
Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. 3.28, 3.31). <b>\$40.00</b> per property					0.00						
Fee for Petition to Revive Unintentionally Abandoned Application (\$1240.00 - Small Entity = \$620.00)					0.00						
<b>TOTAL FEES ENCLOSED =</b>					<b>\$ 990.00</b>						
				Amount to be:							
				refunded	\$						
				Charged	\$						

a. ☒ A check in the amount of \$990.00 to cover the above fees is enclosed.  
b. ☐ Please charge my Deposit Account No. 14-1140 in the amount of \$\_\_\_\_\_ to cover the above fees. A duplicate copy of this form is enclosed.  
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 14-1140. A duplicate copy of this form is enclosed.  
d. ☒ The entire content of the foreign application(s), referred to in this application is/are hereby incorporated by reference in this application.

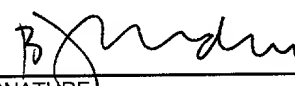
**NOTE:** Where an appropriate time limit under 37 C.F.R. 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

**SEND ALL CORRESPONDENCE TO:**

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**B. J. Sadoff**  
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**36,663**  
 REGISTRATION NUMBER

**May 25, 2001**  
 Date

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re Patent Application of

VEAS et al.

Atty. Ref.: 1721-29

Serial No. Unknown

Group:

Filed: May 25, 2001

Examiner:

For: GP 120 MUTANTS AND BIOLOGICAL APPLICATIONS

\* \* \* \* \*

May 25, 2001

Assistant Commissioner for Patents  
Washington, DC 20231

Sir:

**PRELIMINARY AMENDMENT**

In order to place the above-identified application in better condition for examination,  
please amend the application as follows:

**IN THE CLAIMS**

Please substitute the following amended claims for corresponding claims previously  
presented. A copy of the amended claims showing current revisions is attached.

5. (Amended) Mutants according to claim 2, characterized in that they contain an additional  
mutation according to which F at position 383 is replaced by an alanine.

10. (Amended) Application of the mutants according to claim 1, as vaccine targets.

VEAS et al.  
Serial No. **Unknown**

**REMARKS**

The above amendments are made to place the claims in a more traditional format. Attached hereto is a marked-up version of the changes made to the claims by the current amendment. The attached page is captioned "**Version With Markings To Show Changes Made.**"

Respectfully submitted,

**NIXON & VANDERHYE P.C.**

By: \_\_\_\_\_



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**VERSION WITH MARKINGS TO SHOW CHANGES MADE**

**IN THE CLAIMS**

5. (Amended) Mutants according to [any of claims 2 to 4] claim 2, characterized in that they contain an additional mutation according to which F at position 383 is replaced by an alanine.

10. (Amended) Application of the mutants according to [any of claims 1 to 9] claim 1, as vaccine targets.

09/856707

JC18 Rec'd PCT/PTO 2 5 MAY 2001

Our Ref.: 1721-29

***U.S. PATENT APPLICATION***  
(*ENGLISH TRANSLATED INTERNATIONAL APPLICATION AS FILED*)

***Inventor(s):*** Francisco VEAS  
Martine CERUTTI

***Invention:*** GP 120 MUTANTS AND BIOLOGICAL APPLICATIONS

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***SPECIFICATION***

6/PRTS

09/856707

JC18 Rec'd PCT/PTO 2 5 MAY 2001

gp120 MUTANTS AND THEIR BIOLOGICAL APPLICATIONS

The present invention relates to gp120 mutants and their applications.

It is known that interactions between the CD4 of target cells and HIV gp120 involve conserved gp120 regions, in particular a tryptophan (W) at position 427 according to the amino acid numbering described by Kwong et al (ref. 1 in the bibliography given at the end of this description).

In the article by Missé et al [2], some of whose co-authors are co-inventors in the present application, it was shown that a stable structure, a so-called conserved gp120 structure, contained in the C1 zone, namely the amphipathic  $\alpha 1$  helix, is involved in interactions with the CD4 receptor. In addition, a gp120 construct with no  $\alpha 1$  helix showed that an interaction still remains possible with the target cell, in particular with the CXCR4 receptor, the chemokine receptor on the lymphocytes.

By inducing specific point mutations in the conserved structures of gp120, the  $\alpha 2$  helix in particular, the inventors have identified the essential

role played by these structures in the interactions with target cells, whether directly or indirectly. The trans-conformation of these structures subsequent to a given mutation made it possible, as illustrated in the  
 5 examples, to unmask regions that are normally hidden and which are involved in these interactions, and therefore to make molecules available that are able to act as vaccine targets.

The invention therefore concerns mutants of gp120,  
 10 characterized in that they contain at least one mutation in a region rich in aromatic amino acids, and especially of the  $\alpha 2$  helix of gp120, and optionally the  $\alpha 1$  helix.

The mutants of the invention contain at least one  
 15 mutation, this mutation being located in the gp120 region corresponding to the interaction cavity with CD4.

The invention particularly concerns the gp120 mutants in which W at position 112 is replaced by a  
 20 non-aromatic amino acid such as a serine S.

Said mutation induces a total absence of cell fusion. Cell fusion tests, which are reported below in the example section, therefore show that the W112S substitution annihilates the formation of syncytia  
 25 between HeLa-Tat cells (expressing a gp160 containing this mutation) and HeLa P4 cells. Moreover, a virus containing this same mutation is unable to infect human lymphocytes. Substitution of this same W (bicyclic aromatic residue) by a (monocyclic) aromatic residue  
 30 reduces but does not suppress these functions.

In addition, in said mutants, the recognition by antibodies specific to the CD4 binding site, as defined by Cordonnier et al [3], is greatly reduced.



These results therefore show the capital role played by the conserved  $\alpha 1$  helical structure in fixation to CD4.

In addition to mutation at position 112, such mutants may also comprise a mutation of F at position 383 to alanine, and optionally, of tryptophan at position 427 replaced by a glycine and/or of tryptophan at position 479 replaced by a non-aromatic amino acid, such as serine or isoleucine.

The crystallographic data published in [1] led to visualizing firstly the position of the alpha 1 helix of gp120 in this three-dimensional structure, and secondly to visualizing tryptophan residues.

These data confirmed the results of the biological experiments mentioned above.

In particular, W 112 is located in the hydrophobic pocket described as the gp120-CD4 interaction pocket; this pocket is formed of a "cluster" of aromatic residues which apparently interacts with another aromatic residue, the F43 of CD4. Also, it arises from this study that the tryptophan residues hold a strategic position in this pocket. Therefore any mutation of one of them would destabilize this "cavity" and would prevent any binding with the F43 of CD4.

Other mutants according to the invention optionally also contain, in addition to the above-mentioned mutations in the  $\alpha 1$  helical structure, at least one mutation which is positioned in the gp120 region corresponding to the  $\alpha 2$  helical structure, downstream from the V3 loop of gp120.

The invention particularly concerns the mutant in which W at position 338 is replaced by a serine.

This unique mutation is able to induce a total absence of cell fusion when CXCR4" and cells expressing the W338S muted gp120 are placed in the presence of target CD4" cells.

5       The virus complemented by an enveloped containing the W338S mutation at gp120 is no longer able to infect the cells (see figure 1).

As noted above, since W forms part of the conserved  $\alpha 2$  structure, this amino acid is therefore  
10       found in the second pocket containing aromatic residues and hydrophobic residues.

This mutation therefore suggests that this structure is involved in the interaction with the co-receptors, CXCR4 for example, having regard to  
15       biological results.

The mutants according to the invention, by allowing examination of the role played by conserved structures, provide a new method of approaching interactions between infectious agents-target cells. By  
20       gaining a better understanding of the functionality of the different conserved structures involved in pathogen agent-target cell interactions, it is possible to devise therapeutic or immunizing molecules against HIV.

25       Taking advantage of the identification of these interaction pockets, the invention targets compounds able to mimic CD4 and therefore having inhibitor properties against gp120.

It also targets peptides mimicking the  
30       extracellular loops of co-receptors, able to lodge themselves in said hydrophobic pockets.

The invention also concerns peptides able to mimic the above-mentioned gp120 constant regions and likely

to form candidates as vaccines. The invention therefore provides models for the preparation of such candidates.

In addition, by conducting mutations on the first cavity, it is possible to induce gp120 transformations allowing better exposure of the second cavity, and hence of gp120 as antigen. In advantageous manner, an adapted immune response is therefore obtained against these constant regions that are usually hidden.

Through said transformations, it is also possible to induce dissociations between gp120 and gp41. As shown in the examples given below, no fusion is observed when viruses are used which are complemented with a gp160 containing a mutation at gp120, whereas the glycoprotein responsible for the fusion is gp41. Mutation on gp120 therefore forms a target to prevent fusion of the virus. In this manner key epitopes are made available to prevent infection by HIV.

Other characteristics and advantages of the invention are given in the following examples, in which reference is made to figures 1 to 8 which respectively show:

- figure 1: the results of infectivity tests of HIV-1 pseudovirus complemented with an envelope containing mutations according to the invention at gp120,
- figure 2: the crystalline structure of gp120,
- figure 3: the results of biological activity tests of rgp120, with a rabbit anti-gp120 polyclonal antibody,
- figure 4: the reactivity of rgp120 with AcM CG10,
- figure 5: the reactivity of rgp120 complexed to CD4 with AcM CG10,

- figure 6: the reactivity of rgp120 with AcM 4.8d,
- figure 7: the reactivity of rgp120 complexed to CD4 with AcM 4.8d, and
- 5     - figure 8: the reactivity of rgp120 with AcM 17b.

#### Example: Production and study of gp120 mutants

These gp120 mutants were tested in several  
10 biological systems:

- (i) cell fusion (envelopes muted on the cell surface),
- (ii) viral infections (pseudo-viruses complemented with muted gp120's),
- (iii) analysis of the topology of these muted gp120's made by different
- 15 monoclonal antibodies recognizing different gp120 epitopes in the "CD4 binding site", and (iv) gp120 analysis using crystallographic data.

Those experiments were conducted using the following material and methods:

20

#### • Plasmids, antibodies and pseudoviruses

The pHXB2ENV plasmid (code for the gp160-envelope of HIV-TIIB), the pCMV-rev plasmid (code for the Rev protein controlled by the CMV promoter), and the 902  
25 monoclonal antibody (directed against the V3 loop of gp120) were obtained from NIH AIDS Research and Reference Reagent Program (USA). The pNL4-3env-GFP pseudovirus was supplied by Dana-Farber Cancer Institute, Boston, USA. This plasmid contains the  
30 genome of HIV-1 NL4-3 deleted from the Env gene, and a substitution of the Nef gene by the gene encoding the GFP protein (Green Fluorescent Protein).

#### • Directed mutagenesis in the alpha 1 helix of gp120

For the purpose of examining the W residues present in the alpha 1 helical structure of gp120, the NdeI-HindIII region of the gene coding for gp120 was reconstructed. For this purpose, an oligonucleotide was used in which two restriction sites were inserted (HpaI and HindIII) surrounding the  $\alpha 1$  helix [2]. The  $\alpha 1$  helix of gp120 has two tryptophan residues (W96 and W112) [4]. The HpaI and Hind III sites were used to exchange a wild HpaI-Hind III fragment for a fragment of muted HpaI-Hind III for each construct. Mutations were made either simultaneously on both Ws or on a single tryptophan. Directed mutagenesis was conducted using the overlapping oligonucleotide method between the HpaI and Hind III sites of the pBS plasmid. Table I below only gives those oligonucleotides which contain the desired mutation.

Table I

96W/S	<sup>5</sup> AAC GTG ACA GAA AAT TTT AAC ATG AGT AAA AAT G <sup>3</sup>
112W/S	<sup>5</sup> GAT ATA ATC AGT TTA TCT GAT CAA AGC <sup>3</sup>
96W/1	<sup>5</sup> AAC GTG ACA GAA AAT TTT AAC ATG ATC AAA AAT G <sup>3</sup>
112W/1	<sup>5</sup> GAT ATA ATC AGT TTA ATC GAT CAA AGC <sup>3</sup>
96W	<sup>5</sup> AAC GTG ACA GAA AAT TTT AAC ATG TGG AAA AAT G <sup>3</sup>
112W	<sup>5</sup> GAT ATA ATC AGT TTA TGG GAT CAA AGC <sup>3</sup>

#### • Cell-cell fusion

The expression vectors containing the different gp160 envelopes (pHXB2ENV, 500ng) were co-transfected with 500 ng of pCMV-rec in HeLa-Tat cells. The transfection method used was lipofection (lipofectamine, GIBCO) as described in [2].

pCMV-re is indispensable for activating the expression of the ENV gene present in the pHXB2 ENV

plasmid. The transfected cells were then placed in co-culture with the HeLAP4 cells (CD4-LTR LacZ) as described in [2].

5 • Expression of muted gp120's on the cell surface

The transfection technique used was the same as the one used for cell-cell fusion.

Twenty-four hours before co-transfection the HeLa-Tat cells were placed in wells 3.5 cm in diameter with  
 10 coverglasses (12 mm in diameter). The cells were incubated 48 hours after co-transfection with ACm 902 (directed against the V3 loop of gp120) at a concentration of 10 µg/ml. This incubation was conducted in the presence of 0.1 % sodium azide (NaN<sub>3</sub>)  
 15 and in PBS buffer. After 2 washings in PBS, 0.3 % BSA/0.1 NaN<sub>3</sub>, the cells were then incubated with a mouse anti-IgG antibody labelled with Texas-red (molecular probes). After 3 extensive washings, the cells were fixed for 2 minutes with an ethanol-acetone  
 20 mixture (1:2). After mounting the coverglasses on slides with Mowiol, the cells were observed under confocal microscopy.

• ELFA Assay of p24 (Enzyme Linked Fluorescent Assay)

25 The assay of p24 was conducted with the automated VIDAS HIV DUO system (BioMérieux). This test is based on simultaneous detection of p24 antigenaemia and anti-HIV-1 and anti-HIV-2 antibodies. The principle of the assay associates two immuno-enzymatic reactions with  
 30 fluorescence detection. This test is provided with a disposable cone which serves both as solid phase and as pipetting system. In the lower part of the cone, three synthesis peptides are fixed (gp41, gp36 and a specific

type 0 peptide) conjugated with mouse anti-human IgG Acms labelled with alkaline phosphatase. In the upper part of the cone anti-p24 Acms are fixed. During the assay a rabbit anti-p24 biotinylated antibody  
 5 (conjugated with alkaline streptavidin-phosphatase) is added at the same time as the sample. The substrate, 4-methyl-ombelliferyl-phosphate, is used to measure fluorescence which is proportional to the quantity of anti-HIV Ac and/or Ag p24 present in the sample. The  
 10 reaction occurs in 90 minutes and fluorescence is read with the specific reader for the VIDAS assay (wavelength 450 nm).

• Infections of human lymphocytes with muted pseudo  
 15 viruses obtained by complementation.

293 cells were co-transfected with 10 µg pNL4-3 GFP pseudovirus, 2 µg of pHXB2 ENV plasmid (which may or may not contain the different mutations in SU gp120) and 1.5 µg of pCMV-rev plasmid. The calcium phosphate  
 20 transfection method was used. Forty-eight hours after transfection, the cell supernatants were sampled, filtered and stored at -80°C until use. Measurement of p24 was then made on these different supernatants. Human lymphocytes previously stimulated for 3 days with  
 25 10 µg/ml PHA (Phytohemaglutinine), 50 U of IL-2 in a culture of RPMI 1640-10 % FCS (Foetal Calf Serum), were incubated with the different supernatants of transfected 293 cells (containing an identical p24 concentration).

30 The infecting capacities of the different supernatants were visualized under fluorescence microscopy (expression of GFP protein in the

lymphocytes) and determined by assay of the p24 (periodic follow-up every 3 days) in the culture supernatants of infected lymphocytes.

### 5 • Structural Analyses

The structure of the complex formed by the core of gp120, the first two domains of CD4 and the Fab fragment of the neutralizing antibody 17b were recently determined by X-ray crystallography [1]. This structure  
10 was filed in the protein data base (pdb) under the access code "1qcl". The structural coordinates of this complex were analysed with Insight I software (MSI for Molecular Simulations) on Silicon Grafic O<sub>2</sub>. Visualization and modelling of the domains of gp120 and  
15 CD4 were made using the Discover program (MSI) in an Insight II environment.

### • Results

#### Fusigenic capacities of gp160 envelopes

20 In order to assess the fusigenic capacities of the different gp160 envelopes expressed on the surface of the transfected HeLa-Tat cells, these cells were placed in co-culture with HeLa P4 cells (CD4 LTR LacZ).

The results obtained are given in Table 2 below:

25

Table 2

Mutations in gp 120	Percentage of fusion foci
Wild type	100% (approx. 1500 fusion foci/well)
96W/S and 112W/S	0 %
96W/I and 112W/I	0 %
96W/F and 112W/F	60 %
96W/F	100 %



112W/F	60 %
96W/S	Non-determined
112W/S	0 %
427W/S	0 %
338W/S	0 %
Control	0 %

It is found on examining these results that simultaneous mutations on both tryptophan residues of the alpha 1 helix (W96 and W112) to serine or to isoleucine make the gp160 envelope incapable of inducing syncytia between the cells. The same phenomenon is observed with the gp160 envelope containing the point mutation 112 W/S and/or 338 W/S. On the other hand, the substitution of a tryptophan residue (W) by an aromatic phenylalanine residue (F), having a close hydrophobicity index, reduces the fusigenic capacities of the muted envelopes by about 50 %. It is noticed that, irrespective of the construct, this phenomenon is observed solely when the W112 residue and/or the W338 residue is (are) involved, which denotes the biological importance of this or these residue(s) compared with the W96 group (within the scope of these experiments). All these results were compared with those obtained with the wild gp160 envelope (in which approximately 1500 fusion foci were observed). The negative control was the placing in co-culture of non-transfected HeLa-Tat cells with HeLa P4 cells (no fusion foci were observed).

These results indicate that the W112 residue and/or the W338 residue is or are involved in gp120-target cell interactions, which precede the phenomenon of cell fusion.

• Expression of gp120 proteins on the cell surface.

gp120 was detected by Acn 902, directed against the V3 loop of gp120. The latter was then developed with a mouse anti-IgG coupled with Texas Red. It is found that both the wild gp120 and the 112W/S gp120 are expressed on the surface of the cells. The incubation of the 902 antibody with non-transfected HeLa-Tat cells gives no signal.

• Infective capacities of complemented pseudo-viruses.

Figure 1 shows the changes in the p24 level in the supernatants of lymphocytes (previously activated with 10 µg/ml PHA and 50 U/ml of IL2) placed in contact with:

- the pseudovirus complemented with wild type gp120 (tracing with ●),
- the pseudovirus complemented with muted gp120 (112W/S gp120) (tracing with ○),
- the pseudovirus complemented with muted gp120 (gp120 338W/S) (tracing with ■)

In the first case, the p24 level is firstly reduced and then increases considerably (on and after the 10<sup>th</sup> day after infection), which proves the presence of a *de novo* synthesized p24, the product of a viral infection.

In the second case, the p24 level reduces gradually to disappear completely on the 20<sup>th</sup> day (after contacting). No cell infection therefore occurred due to the 112W/S mutation in gp120. This fact was confirmed by the absence of expression of the GFP protein in the cells incubated with the muted virus 112

W/S and/or 338 W/S (visualization under fluorescence microscopy).

The lack of cell infection observed in this latter case may be explained by the absence of any syncytia  
5 formation previously visualized with cells expressing gp120 W/S and/or gp120 338 W/S on their surface.

• Three-dimensional analysis and structural locating of the conducted mutations

10 The crystallographic structure of the gp120-CD4-17b Fab complex has recently been determined [3] with a resolution of 2.5 Å (Figure 2).

The coordinates of this structure were analysed using Insight II software, with which it was possible  
15 to place the amino acids in a three-dimensional context with much higher resolution than with the predictive models which prompted our approach.

Analysis of the crystallographic structure of the gp120-CD4 complex shows a reduced number of residues  
20 forming the interface between the two proteins. The majority interaction between gp120 and CD4 is located at a hydrophobic pocket, involving the W427 amino acid of gp120 and the F43 residue of CD4 (Wyatt et Coll. 1998).

25 In addition, as shown in Figure 3, W112 is strategically located in this cavity rich in aromatic residues and interacts directly with the F43 of CD4. F43 apparently places itself in the middle of this pocket and would seem to interact directly with W112.  
30 The interaction of W112 with F43 and W427 apparently makes it possible to stabilize the entire hydrophobic pocket. It is to be noted that W112 and W427 are located at a distance of 5 Å or less from each other,

which indicates that these two tryptophan residues are likely to interact strongly.

This interaction could occur through a stacking effect of the aromatic cores between these two residues or by dipole-dipole binding. Also, these distances may in fact prove to be much shorter taking into account the dynamic aspect of *in vivo* molecular interactions.

Crystalline analysis also led to discovering that W338 of the  $\alpha 2$  helix is likely to play a major role in the stability of the second cavity (out of a total of 2 cavities included inside gp120), formed of the hydrophobic and aromatic residues. Under these conditions, this cavity may be considered to be capital in the interactions with gp120 and the co-receptors, CXCR4 for example.

- Experiments identifying the involvement of W386S mutation on the HIV-1 gp120 of the HXB2 clone of HIV-III<sub>B</sub> in terms of its structure and function.

FUNCTION: fusogenic capacities of gp120 expressed on the surface of transfected cells and infective capacities of viruses having envelopes with muted or non-muted gp120s

No observation is made :

- of any fusion foci in the fusion tests between HeLa-P4 cells (CD4+/CXCR4+) and HeLa  $\Delta 20$  (Tat) cells transfected with the envelope of the pHXB2 clone of the HIV-1 III<sub>B</sub> strain (see table 2). The positive control with the wild envelope gives 1800 to 2000 foci. The negative control was made with an envelope

of the MoMuLV retrovirus which does not produce any fusion foci under these conditions.

- no infection of lymphocyte cells activated with PHA (Phytohemagglutinine) and with interleukin 2 (IL-2) by viral pseudotypes obtained after complementing the genome of HXB2 ENV- (pNL4.3ΔENV) and the envelope (pHXB2 ENV) with W338S gp120, and the envelope inside 293T cells transfected with the 2 expression vectors.
- The positive control was formed of pNL4.3 ΔENV and pHXB2 ENV (wild type). The negative control was formed by a supernatant of the cells transfected only with the expression vectors pHXB2 ENV.

#### 15 STRUCTURE:

##### ELISA assay: Fixing of gp120 only to soluble CD4

- Figures 4 to 8 give the results of the reactivity of wild rgp120, gp120ΔaHX1 and gp120Δ3 W338S, with AcM CG10 (figure 4), AcM 4.8d (figure 6) and with AcM 17b (figure 8): in these assays, the rgp120s were deposited at a concentration of 1 µg/ml. The AcM was deposited at the concentrations shown on the abscissa. Figures 5 and 7 give the reactivity of these rgp120s complexed to CD4 with AcM CG10 (figure 5) and AcM 4.8d (figure 7): in these assays the rgp120s were deposited at a concentration of 1 µg/ml. The CD4 concentration was 20 µg/ml and the AcM was deposited at the concentrations shown on the abscissa.

- This mutation induces a trans-conformation which allows better exposure to the monoclonal antibodies 17b, 4.8d and G10 directed against the CD4i epitopes (induced CD4) (Figures 4, 5, 6, 7 and 8) which are

epitopes that are unmasked after the fixing of gp120 to CD4. This trans-conformation was able to be detected in an ELISA system which was conducted as follows:

1. The bottom of the wells is coated with soluble CD4 (reference NIH) or with the antibody Aalto D7324 (which is a goat antibody) directed against a linear peptide of the C-terminal region of gp120 (this antibody does not induce the trans-conformation to obtain unmasking of the CD4i epitopes).
2. After saturation with 3 % BSA, gp120 is incubated with CD4 or with the antibody D7324 at 37°C for 1 hour.
3. After 2 washings, one of the anti-CD4i AcM's (17b or 4.8d or CG10) is incubated with the previously attached gp120 (wild type or mutant) for 1 hour at 37°C.
4. The CD4i antibodies are developed with an anti-IgG antibody conjugated with peroxylase (POD).
5. The intensity of the reaction is read in the ELISA reader after halting the reaction.

The background noise shown by the controls (cells without gp120) is withdrawn from assay point results.

Remark: a control was made with gp120's detected by a rabbit polyclonal antibody directed against gp120 (figure 3).

These assays made with ELISA made it possible to verify that the anti-CD4i AcM's 17b and 4.8d indeed better recognize wild gp120 and muted gp120 when the latter are associated with soluble CD4, allowing unmasking of these epitopes. Although AcM CG10 does not at all recognize wild gp120 non-complexed to CD4, this

AcM recognizes muted gp120 without it being complexed to CD4.

Interpretation:

- 5       The W338S mutation on gp120 induces the unmasking of the CD4i epitopes, in particular the epitopes recognized by AcM CG10.

10       This result is of importance since this mutation makes this mutant a good vaccine candidate exposing masked epitopes in infective viruses.

FACS Assay: Simultaneous fixing of gp120 to at least 2 receptors present on the cell surface of the CEM lymphocyte line: CD4 and CXCR4.

- 15       In order to check that gp120 fix themselves to CD4 and CXCR4 respectively, the following assay was conducted: the CEM cells were firstly incubated with gp120, then incubated with monoclonal antibodies against the CD4 receptor (AcM OKT4a FITC supplied by
- 20   ORTHO Diagnostic) and against the CXCR4 receptor (AcM 12GS supplied by Pharmingen) and then with appropriate mouse anti-IgG antibodies.

25       Table 3 gives the average fluorescence intensity of gp120 fixed to the CEM cells CD4+/CXCR4+ recognized by anti-CD4 AcM's.

Table 3

Anti-CD4i AcM antibodies	AVERAGE FLUORESCENCE INTENSITY OF GP120 FIXED TO CEM CELLS CD4+ / CXCR4+		
	without gp120	wild gp120	W338S gp120
4.8d	5.65	76.775	208.76
17b	5.21	70.48	175.575
CG10	5.14	7.15	8.49

No labelling was observed which means that gp120 is effectively fixed to CD4 and to CXCR4.

5 The CEM cells cultured in RPMI 10 % FCS were washed with a wash buffer PBS/0.3%BSA/0.02% Na-azide (WB) and placed in an incubation buffer PBS/3%BSA/0.02% Na-azide (IB) so as to place them in contact with wild and mutant gp120 for 1 hour at 37°C.

10 The cells were washed twice with the WB then incubated in IB with one of the anti-CD4i antibodies (17b, 4.8d or CG10) at a concentration of 10 µg/ml for 1 hour at 25°C. The cells were then washed twice with WB, then incubated 45 minutes at 25°C in IB with an  
15 appropriate anti-IgG antibody conjugated with FITC or PE. After 3 washings with WB, the cells were replaced in suspension in IB and analysed under flow cytometry (FACSsort). 10 000 cells could be analysed one by one.

20 The results are given in figure 5. The 17b and 4.8d antibodies well recognize the muted gp120 but only scarcely recognize wild gp120. On the other hand, AcM CG10 no longer has access to its recognition site either on wild gp120 or on muted gp120.

25



### Interpretation

If AcM CG10 no longer has access to its site but on the other hand AcM 17b and 4.8d do have access to this site, this means that the gp120 epitope strongly  
 5 involved in the fixing of gp120 to the CXCR4 co-receptor is especially the one which is recognized by CG10.

### Conclusion

10 W338S mutation on gp120 induces a trans-conformation which prevents fusion and infection. This mutation prevents fusion when the envelope is expressed on the surface of transfected cells, and prevents  
 15 complemented virus. In addition, this trans-conformation allows unmasking of masked sites on wild gp120, due essentially to the fact that muted gp120 is recognized by AcM CG10 without this gp120 being complexed to CD4. The fact that this same AcM CG10 no  
 20 longer has access to its epitope when this protein is associated with CD4 and CXCR4, and the fact that the anti-CD4i antibodies do not interfere with CD4, implies that it is the epitope recognized by AcM CG10 on gp120 which is involved in the fixing to CXCR4. Such mutant  
 25 forms a candidate for conducting immunisations for the purpose of protecting against HIV infection.

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2. Missé D., Cerruti M., Schmidt I., Jansen A., Devauchelle G., Jansen F. and Véas F., 1998. Dissociation of the CD4 and CXCR4 binding properties of human immunodeficiency virus type 1 gp120 by deletion of the first putative alpha-helical conserved structure. *J. Virol* 72:7280.
3. Cordonnier A., Montagnier L., and Emmerman M. 1989. Single amino-acid changes in HIV envelope affect viral tropism and receptor binding. *Nature* 340:571.
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English translation of annex to IPER

CLAIMS

1. HIV gp120 mutants characterized in that they contain in their  $\alpha 2$  structure or in both  $\alpha 2$  and  $\alpha 1$  structures, a mutation of one or more aromatic amino acids, and in that this mutation gives rise to a loss  
5 in the infective properties of the mutants relative to wild gp120.

2. Mutants according to claim 1, characterized in that the muted amino acid or acids are positioned in the gp120 region corresponding to the interaction  
10 cavity with CD4, such as identified by crystallography.

3. Mutants according to claim 2, in which W at position 112 is replaced by a non-aromatic amino acid.

4. Mutants according to claim 3, characterized in that the non-aromatic amino acid is chosen from among a  
15 serine or an isoleucine.

5. Mutants according to any of claims 2 to 4, characterized in that they contain an additional mutation according to which F at position 383 is replaced by an alanine.

20 6. Mutants according to claim 5, characterized by a mutation of tryptophan at position 427 to glycine, and/or of tryptophan at position 479 to serine.

7. Mutants according to claim 1, characterized in that they contain at least one mutation which is located in the gp120 region corresponding to the  $\alpha 2$  helical structure, downstream from the V3 loop of gp120.

8. Mutants according to claim 7, characterized in that W at position 338 is replaced by a non-aromatic amino acid.

9. Mutants according to claim 8, characterized in that the non-aromatic amino acid is chosen from among a serine or an isoleucine.

10. Application of the mutants according to any of claims 1 to 9, as vaccine targets.

11. Application according to claim 10, characterized in that as antigenic vaccine target, a mutant is used in which W at position 338 is replaced by a non-aromatic amino acid.

12. Application according to claim 10, characterized in that the non-aromatic amino acid is chosen from among a serine or an isoleucine.

13. Method for suppressing the infectivity of an HIV gp120, characterized in that the  $\alpha 2$  structure, or both the  $\alpha 2$  and  $\alpha 1$  structures, are muted on one or more aromatic amino acids.

14. Method according to claim 13, characterized in that mutation in the  $\alpha 1$  structure is conducted in the gp120 region corresponding to the interaction cavity with CD4, such as identified by crystallography.

Figure 1

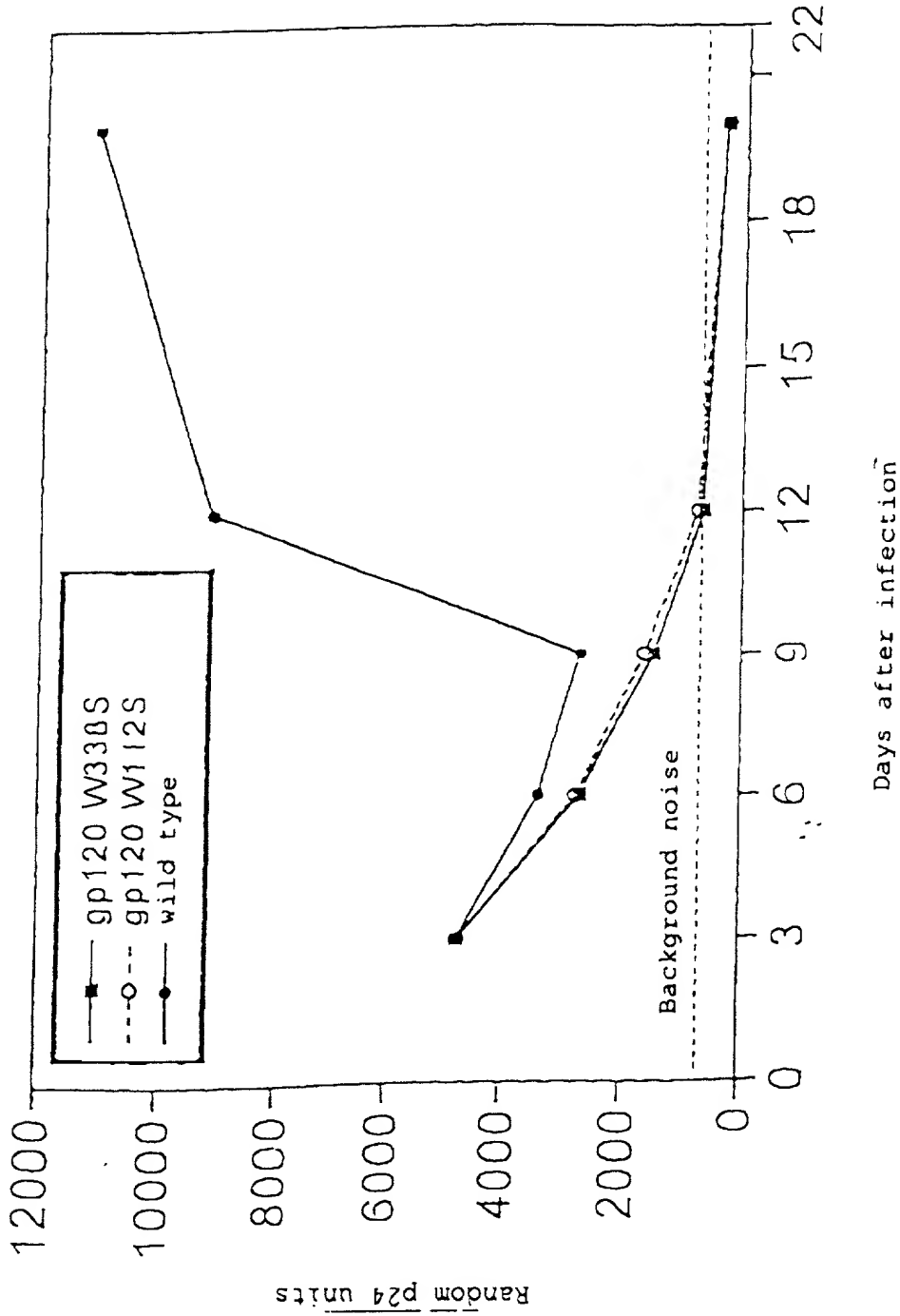


Figure 3

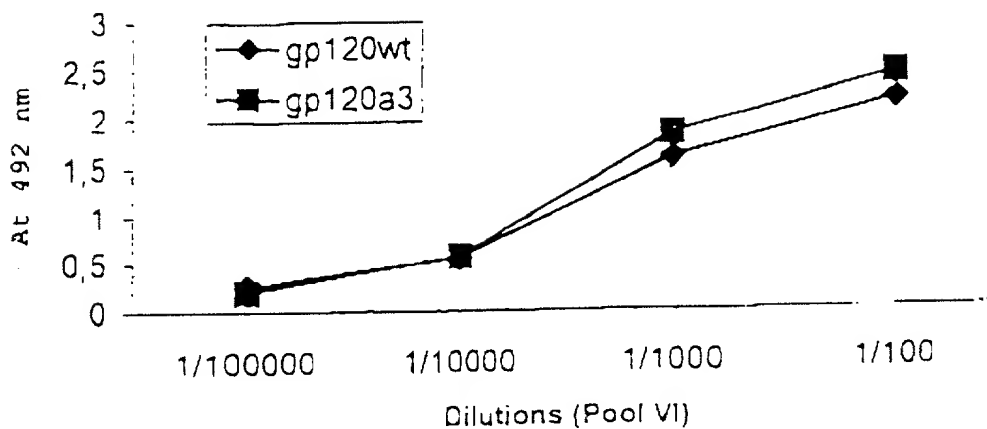
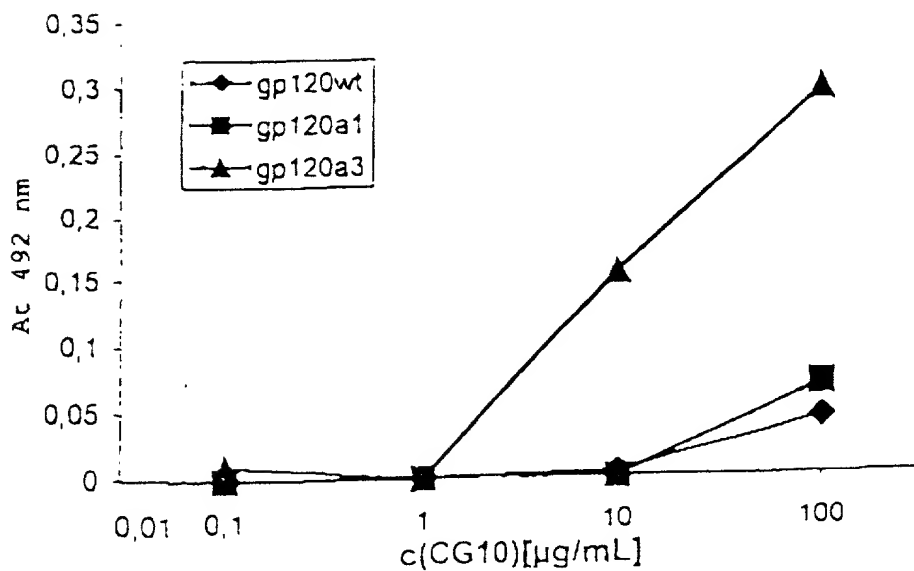


Figure 4



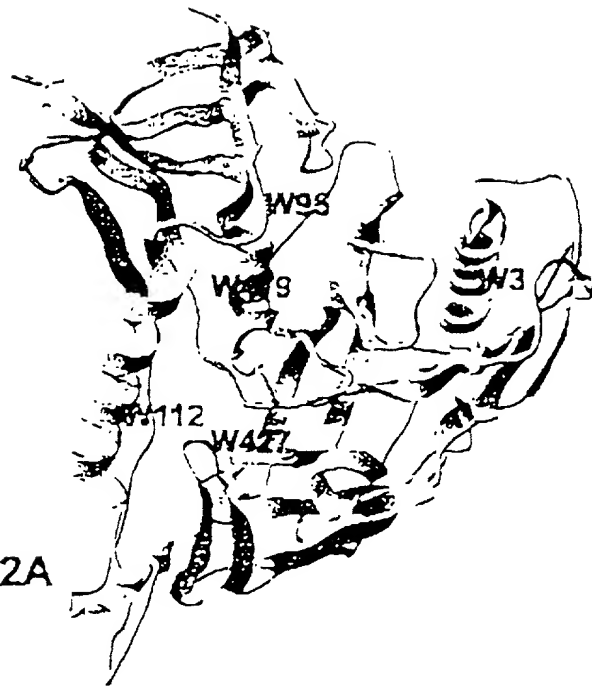


Fig. 2A

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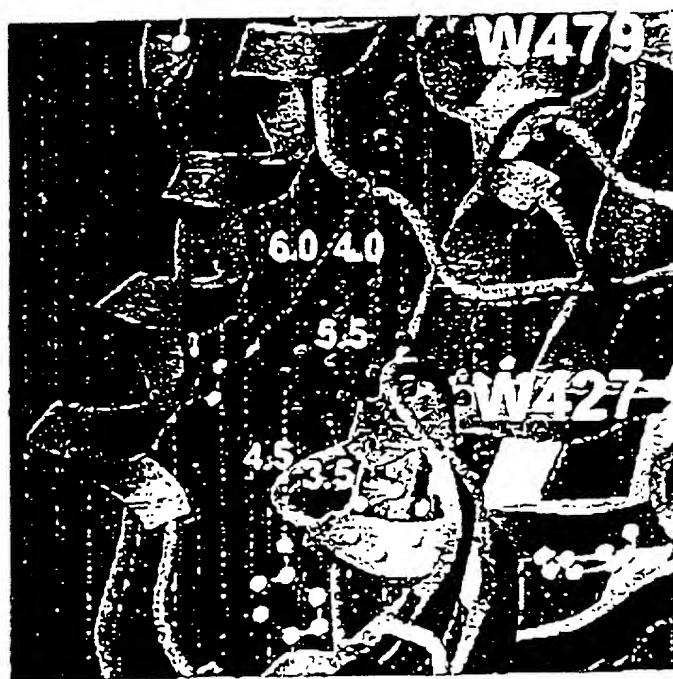


Fig. 2B



Figure 5

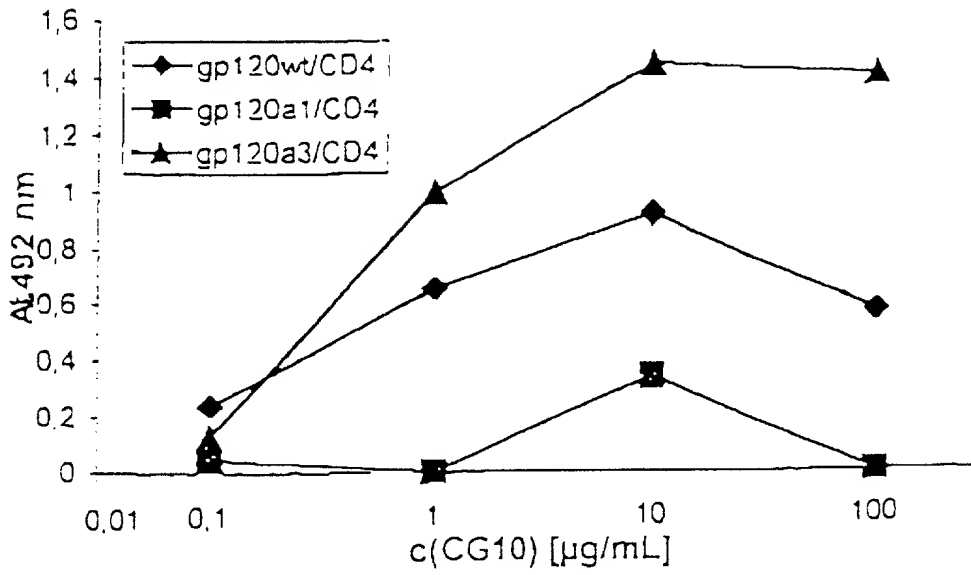
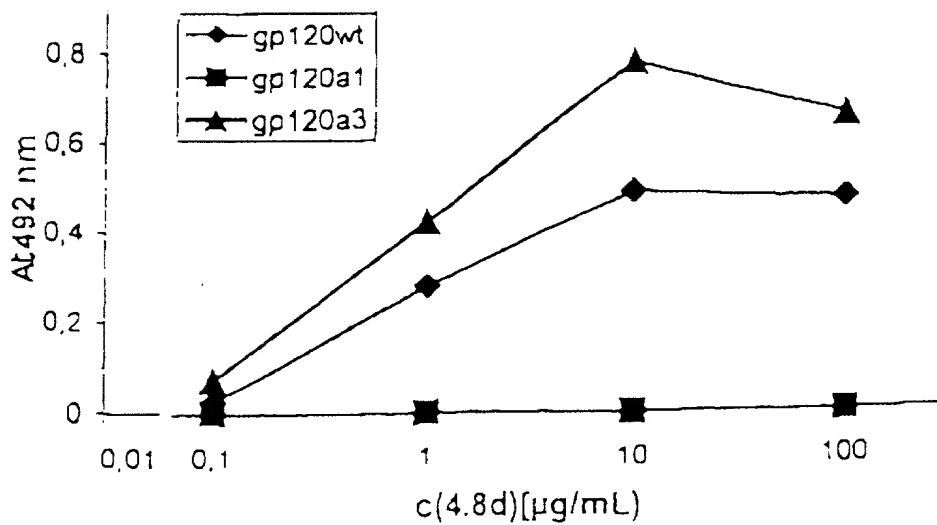


Figure 6



09/856707

Figure 7

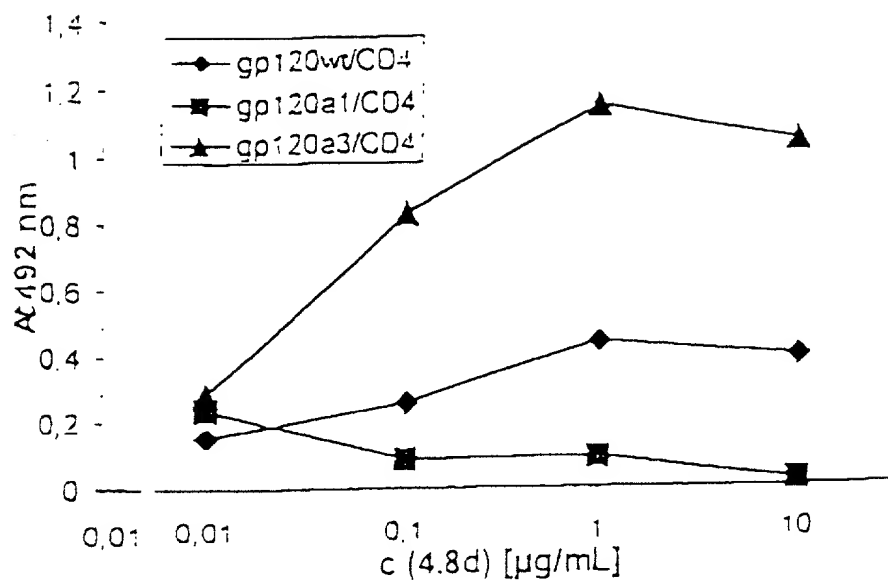
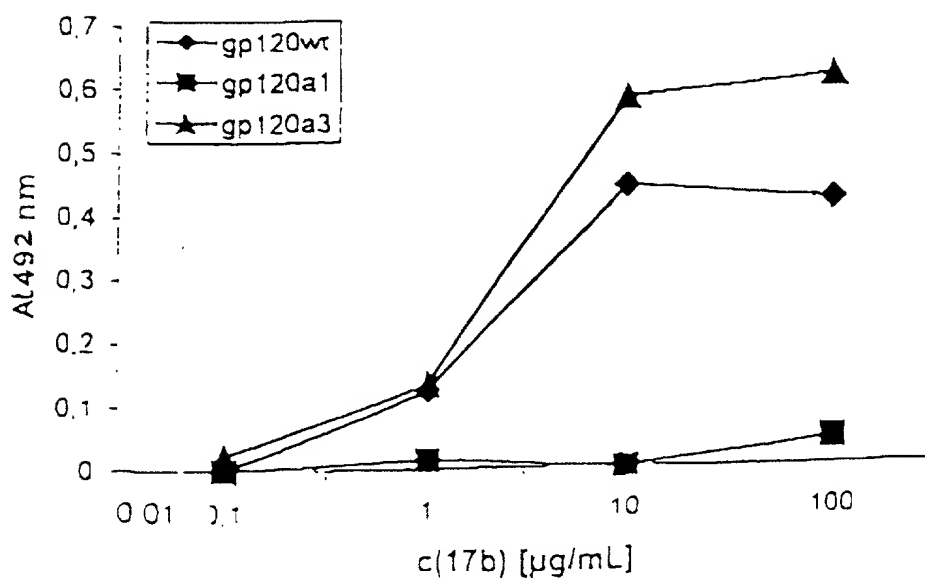


Figure 8



**RULE 63 (37 C.F.R. 1.63)**  
**INVENTORS DECLARATION FOR PATENT APPLICATION**  
**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

As a below named inventor, I hereby declare that my residence, mailing address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**GP 120 MUTANTS AND BIOLOGICAL APPLICATIONS**

the specification of which (check applicable box(es)):

☐ is attached hereto  
☐ was filed on \_\_\_\_\_ as U.S. Application Serial No. \_\_\_\_\_ (Atty Dkt. No. 1721-29)  
☒ was filed as PCT International application No. PCT/FR99/02949 on 29 November 1999  
 and (if applicable to U.S. or PCT application) was amended on December 20, 2000

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose to the Patent Office all information known to me to be material to patentability as defined in 37 C.F.R. 1.56. I hereby claim foreign priority benefits under 35 U.S.C. 119/365 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed or, if no priority is claimed, before the filing date of this application:

Priority Foreign Application(s).

Application Number	Country	Day/Month/Year Filed
98 14997	France	27 November 1998

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below

Application Number	Date/Month/Year Filed
--------------------	-----------------------

I hereby claim the benefit under 35 U.S.C. 120/365 of all prior United States and PCT international applications listed above or below.

Prior U.S./PCT Application(s):

Application Serial No.	Day/Month/Year Filed	Status: patented pending, abandoned Pending
PCT/FR99/02949	29 November 1999	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon. And on behalf of the owner(s) hereof, I hereby appoint NIXON & VANDERHYE P.C., 1100 North Glebe Rd., 8<sup>th</sup> Floor, Arlington, VA 22201-4714, telephone number (703) 816-4000 (to whom all communications are to be directed), and the following attorneys thereof (of the same address) individually and collectively owner's/owners' attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent: Larry S. Nixon, 25640; Arthur R. Crawford, 25327; James T. Hosmer, 30184; Robert W. Fans, 31352; Richard G. Basha, 22770; Mark E. Nusbaum, 32348; Michael J. Keenan, 32106; Bryan H. Davidson, 30251; Stanley C. Spooner, 27393; Leonard C. Mitchard, 29009; Duane M. Byers, 33363; Jeffrey H. Nelson, 30481; John R. Lastova, 33149; H. Warren Burnam Jr. 29366; Mary J. Wilson, 32955; J. Scott Davidson, 33489; Alan M. Kagen, 36178; Robert A. Molan, 29834; B. J. Sadoff, 36663; James D. Berquist, 34776; Updeep S. Gill, 37334; Michael J. Shea, 34725; Donald L. Jackson, 41090; Michelle N. Lester, 32331; Frank P. Presta, 19828; Joseph S. Presta, 35329; Joseph A. Rhoa, 37515; Raymond Y. Mah, 41426; Chris Comenzis, 31097. I also authorize Nixon & Vanderhye to delete any attorney names/numbers no longer with the firm and to act and rely solely on instructions directly communicated from the person, assignee, attorney, firm, or other organization sending instructions to Nixon & Vanderhye on behalf of the owner(s).

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